Life Sciences

Development Of An Aptamer-Based MRI Contrast Agent For Thrombin Detection

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Aptamer technology is an emerging tool that utilizes engineered strands of DNA or RNA oligomers to bind a target with high affinity. Because of its low toxicity, aptamers can have many applications in biotechnology and clinical research. Conventional magnetic resonance imaging (MRI) contrast agents (CA) are administered as ions bound to a chelator. This conventional imaging method lacks specificity to areas of interest, compromising precision. Here, Daphnee Dubouchet-Olsheski aims to build specificity into an MRI CA used to image blood clots. For this, a thrombin-specific aptamer is conjugated to a CA chelator, tethering the contrast agent to thrombin. Daphnee explores the initial steps required to synthesize chelator-aptamer candidates that will be used in future studies. Aptamers integrated into medical technology can provide an innovative solution to sharpen the contrast of conventional MRI.

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Thrombin is an enzyme that converts soluble fibrinogen into insoluble strands of fibrin (which works with platelets to form a blood clot). MR imaging of thrombin could be useful in the imaging of blood clots (thrombus and embolus). An MRI contrast agent can be made more specific for a certain protein by conjugating (joining) it to an aptamer. An aptamer is a synthesized single-stranded DNA, targeted (through a process called SELEX) to a specific protein. The development of a targeted MRI contrast agent could enhance the diagnostic value of the obtained MR images. Through screening two series of contrast agents, DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) and DTPA (diethylenetriaminepentaacetic acid), this project hopes to find the best system for measuring thrombin in serum. This use of aptamer technology (and DNA synthesis) appears to be a unique method of targeting thrombin rather than the more invasive angiography and angioscopy procedures. The first phase of this project involved the synthesis of aptamer-DOTA and aptamer-DTPA conjugates. Progress towards the development of these conjugates is presented. Upon successful synthesis of the aptamer conjugates, they could be screened to find the best system for measuring thrombin in serum.

La thrombine est une enzyme qui convertit le fibrinogène soluble en brins de fibrine insoluble (ce qui permet aux plaquettes de former un caillot de sang). L'imagerie à résonance magnétique (IRM) de la thrombine pourrait être utile pour l'imagerie de caillots sanguins (thrombus et embolie). Un agent de contraste IRM peut être conçu pour viser plus spécifiquement une certaine protéine par voie de conjugaison (liaison) à un aptamère. Un aptamère est simple brin d'ADN synthétisé, qui cible (par le processus SELEX) une protéine spécifique. L'élaboration d'un agent de contraste IRM ciblée envers une protéine particulière pourrait améliorer la valeur diagnostique des images IRM obtenues. Grâce à un dépistage de deux séries d'agents de contrastes, DOTA (acide 1,4,7,10-tétraazacyclododécane-1,4,7,10-tétraacétique) et DTPA (acide diéthylènetriaminepentaacétique), ce projet vise à trouver la meilleure méthode afin de mesurer la thrombine dans le sérum. L'utilisation de cette technologie avec

aptamère (et avec la synthèse de l'ADN) semble être une méthode unique afin de cibler la trombine de manière moins invasive que les procédures d'angiographie ou d'angioscopie. La première phase de ce projet consistait de la synthèse du conjugué de l'aptamère DOTA et de l'aptamère DTPA. Le progrès vers l'élaboration de ces conjugués est présenté dans cet article. Dès la réussite de la synthèse des conjugués d'aptamères, ces derniers pourraient être en mesure d'être évalué afin de trouver la meilleure méthode pour mesurer la thrombine dans le sérum.

Background

Magnetic resonance imaging (MRI) is a common medical technique used to obtain high-resolution three-dimensional images of body tissue. Signal contrast in MR images depends on the "relaxation" of *in vivo* water, which can be increased by administering a contrast agent (CA). Many clinical contrast agents contain the paramagnetic Gadolinium(III) ion (Gd(III)).[1] Gd(III) cannot be administered as a free ion because of its high toxicity, so it is typically chelated with a compound such as diethylenetriaminepentaacetic acid (DTPA) or 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) for use as an MRI agent. Despite the need for specificity, most CAs - including those based on DOTA and DTPA - circulate throughout the whole body and do not discriminate between various organs and tissues. Effectively targeted MRI CAs would be very valuable, as lower doses would be sufficient and still provide sharper contrast in MR images compared to non-specific CAs. Some target-specific MRI CAs are already in clinical use; for instance, MS-325 (Ablavar®) is used to image blood vessels.

A target-specific MRI CA can be prepared by linking a Gd(III) chelate to a receptor molecule that specifically recognizes the tissue of interest. While antibodies are commonly used as recognition molecules, aptamers are emerging as a more robust alternative. Aptamers are single-stranded DNA or RNA sequences that fold into distinct nanoscale shapes capable of binding specifically to a target molecule. Bernard and colleagues recently published a proof-of-concept study, in which an aptamer was conjugated to a DTPA chelate. This conjugate was found to improve MRI contrast by about 30% in the presence of a target protein.[2]

Purpose

Thrombin is an enzyme that converts soluble fibrinogen into insoluble strands of fibrin, which works with platelets to form a blood clot. MR imaging of thrombin could be useful in visualizing blood clots (thrombus and embolus). In this study, the objective was to develop a practical MRI contrast agent for imaging thrombin and blood clots by conjugating a specific aptamer to a chelate. A 29 base long DNA aptamer, isolated by Kubik *et al*, has been shown to bind to thrombin in blood clots and was used in this study of MRI contrast agents.

Materials and Methods

Synthesizing the Aptamer-Chelate Conjugate

The aptamers were synthesized by standard phosphoramidite chemistry on a MerMade 6 DNA synthesizer. All amidites (bases) were prepared using specified volumes of acetonitrile for each base. The amidites and the synthesis columns were then loaded onto the MerMade synthesizer and the amino modifier (NH2) was added. The file was then set up to specifically synthesize 5'-AGTCCGTGGTAGGGCAGGTTGGGGTGACT-3'.Two series of thrombin aptamer conjugates were prepared: one using the DTPA chelator, and the other using the DOTA chelator. These conjugates were prepared through the reaction between 5'-amino-modified aptamers and isothiocyanate-modified chelators to form a thiourea linkage (Figures 1 and 2). After the DNA synthesis was complete, the synthesis columns were washed in 10% diethylamine in anhydrous acetonitrile. The DNA was then washed with a deblocking reagent (2% dichloroacetic acid in dichloromethane). The beads were then transferred to a microcentrifuge tube and suspended in anhydrous dimethyl sulfoxide (DMSO) containing 15 µmol of either DTPA or DOTA chelates. The chelate reaction was allowed to react overnight. The beads were finally washed with DMSO and reacted for 24 hours with ammonium hydroxide before being dried on the speed vacuum.

Purifying the Aptamer-Chelate Conjugates

Following their synthesis, the conjugates were purified by polyacrylamide gel electrophoresis (PAGE). PAGE is a method of purifying and analysing DNA according to the size and charge of the molecule.

A 12% polyacrylamide gel was prepared by dissolving 31.5 g of urea in 23.5 mL acrylamide/bisacrylamide stock solution, 14 mL of water and 15 mL of 5X TBE buffer. The solution was heated to 37°C, then filtered. 0.45 mL of 10% ammonium persulfate and 0.035 mL of TEMED were added to this remaining solution; the solution was swirled quickly in the flask and poured into a gel-casting apparatus. The gels were allowed to polymerise for 30 minutes before samples were loaded and run. To prepare the samples, the DNA was dissolved in an equivolume of water and formamide, and the samples were heated to 55°C for 5 minutes. The dark band in the gel containing the oligonucleotide was recovered. Finally, the gel/DNA solution was filtered, dried using a lypholizer, and desalted.

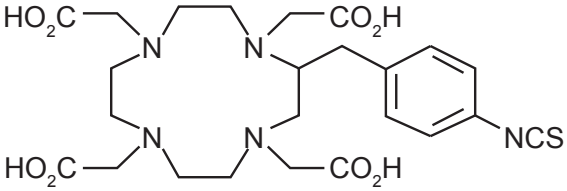

Figure 1A. SCN - DOTA chelate

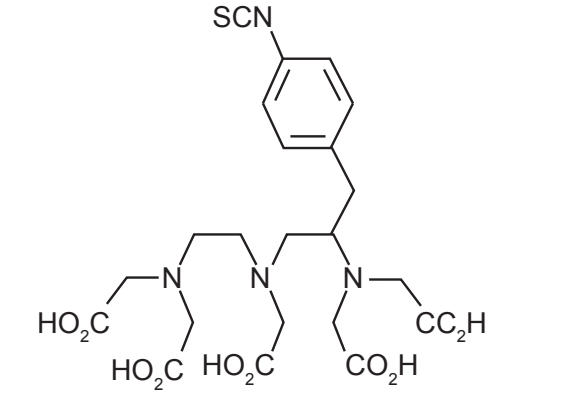

Figure 1B. SCN - DTPA chelate

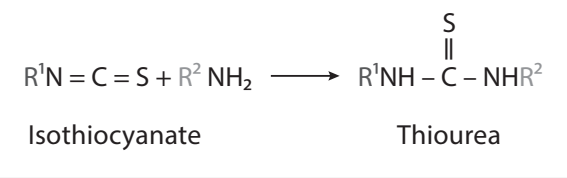

Figure 2. Scheme for the preparation of aptamer-MRI chelate conjugates; Thiourea conjugate reaction: where R^1 = the chelator and R^2= the aptamer.

Analyzing the Aptamer-Chelate Conjugates

The modified chelators were quantified by UV Vis and sent for mass spectrometry. A separate sample was loaded with Gd(III) ions and subjected to the Xylenol orange test for loading efficiency.

Results

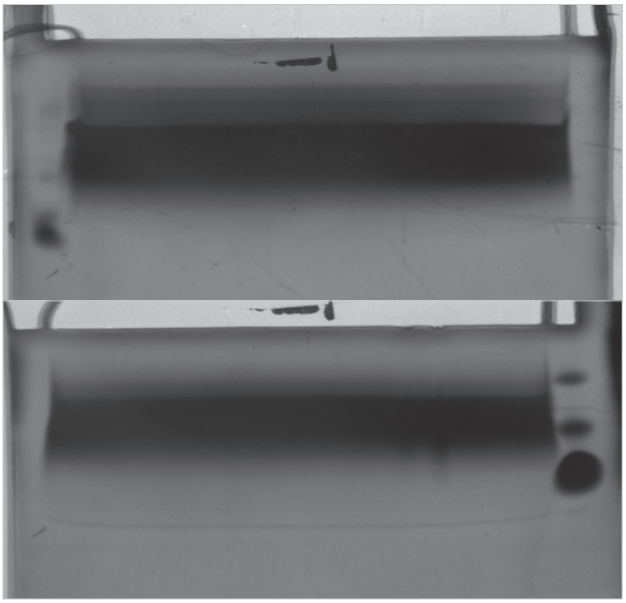

Figure 3. PAGE purification; top: aptamer-DOTA conjugate, bottom: aptamer-DTPA conjugate.

Aptamer DNA was Successfully Synthesized

The thick, dark bands in Figure 3 suggest that the DNA was successfully synthesized. The bands containing DNA were cut out and the DNA-chelate conjugates were extracted and desalted to purify the sample of extra salts and urea. Subsequently, the DNA was quantified by UV-Vis Spectroscopy.

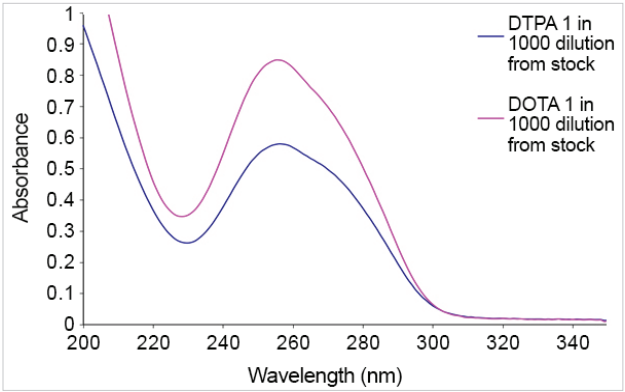

Figure 4. UV-Vis absorbance spectrum of DNA for quantification of the DNA-chelate conjugates.

The presence of DNA is indicated by a peak at ~260 nm (Figure 4). Using the Beer-Lambert Law, we found that 296 nmol and 193 nmol of the aptamer-DOTA and aptamer-DTPA conjugates were respectively synthesized. 3 nmol of each aptamer-chelate conjugate was sent for mass spectrometry to verify if the reaction was successful.

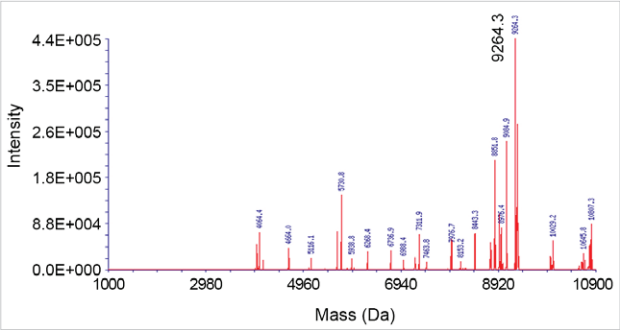

Figure 5. Mass spectrometry results: DOTA first round of MS

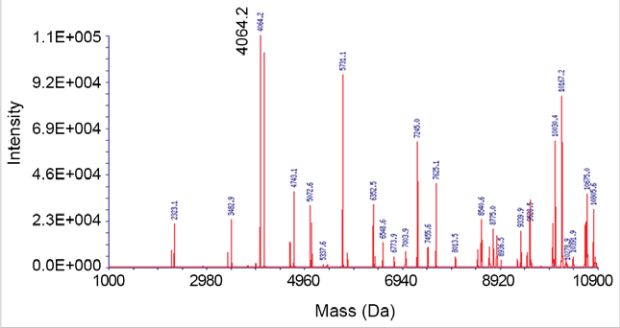

Figure 6. Mass spectrometry results: DTPA first round of MS

Trace Amounts of the Aptamer-Chelate Conjugates were Synthesized

To determine whether the aptamer-chelate conjugate was successfully synthesized, the sample was assessed using mass spectrometry. After comparing the masses observed in the spectra to the theoretical masses of the aptamer-DTPA and aptamer-DOTA conjugates, it was evident that the conjugates had not been synthesized efficiently since the major products (peaks at 9264.3 g/mol and 4064.2 g/mol) were too small. The theoretical mass of the the aptamer-DOTA conjugate is 9952.9 g/mol and the theoretical mass of the aptamer-DTPA conjugate is 9914.8 g/mol. Small traces of the conjugates may have been synthesized successfully; however, a side reaction with DMSO would have resulted in an unstable final product. The peak at ~10030 g/mol in both spectra is evidence that the side reaction occurred.

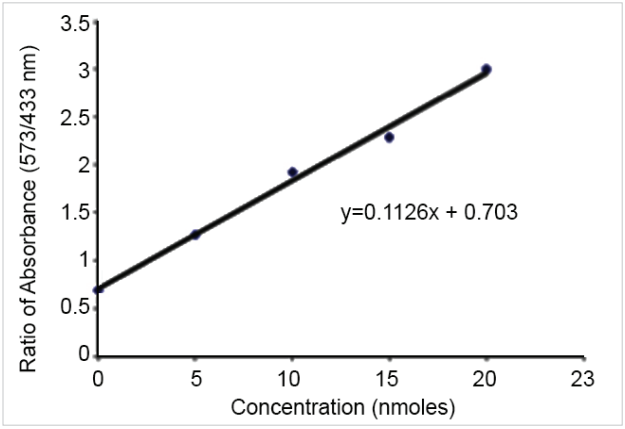

Figure 7. Calibration curve from Xylenol Orange test.

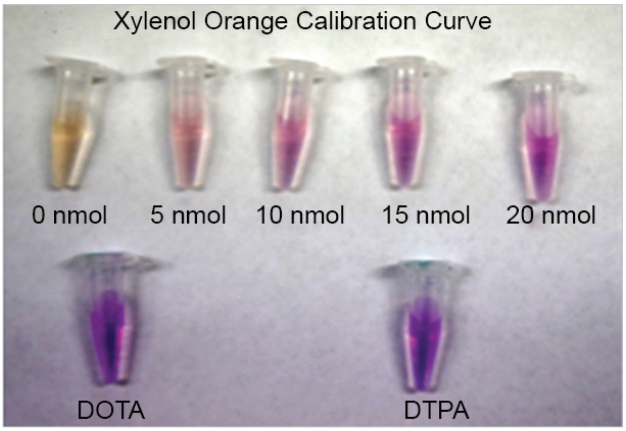

Figure 8: Eppendorf tubes containing Xylenol Orange test reactions.

Gd(III) Loading Efficiencies were Less Than 80%

After determining the molar quantities of the aptamer-chelate conjugates, an equimolar amount of Gd(III) was reacted with them. The Xylenol Orange test was used to determine the concentration of Gd(III) that reacted with the conjugate (Figure 5). The absorbance ratios of the flow-through from the Gd(III) incubations were compared with absorbance ratios of known concentrations of Gd(III) (Figure 7 and 8). We found that the loading efficiencies of the chelates were less than 80%. For the loading efficiency to be higher, more Gd(III) should be added.

Discussion

Harnessing nanotechnology for use in the field of medicine is a burgeoning reality. Here, its use as a diagnostic technique holds promise as some of these

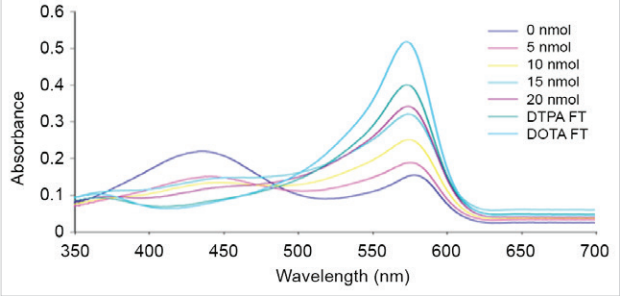

Figure 9. UV-Vis absorbance spectrum of Xylenol Orange test calibration curve.

techniques are in advanced stages of testing and are currently being used today. MR imaging is a valued alternative to the conventional angiography and angioscopy procedures to detect coronary thrombosis and the computed tomography pulmonary angiogram to detect pulmonary embolisms. This technology can be enhanced through the use of aptamer technology. A contrast agent developed using nanotechnology would specifically target the enzyme, thrombin, thus allow for precise imaging of blood clots.

The amino-modified aptamer was successfully synthesized, purified and, subsequently, quantified in relatively high yield and purity compared to previous studies. Previous studies produced around 20 nmol, whereas here, 96 nmol and 193 nmol were produced.

The initial phase of this research generated results that require refining. When conjugating the aptamer and the chelate, a side reaction with DMSO resulted in an unstable final product. The peak at ~10030 g/mol in both spectra was evidence that the side reaction occurred. Future work will involve reacting the DNA once more with the DTPA/DOTA chelate to form the aptamer-chelate conjugate using, instead, an aqueous buffer (versus DMSO which is organic).

We plan to screen two series of contrast agents, DOTA and DTPA, each conjugated to a thrombin-specific aptamer, to find the best system for targeting thrombin in serum. This use of aptamer technology promises to be less invasive than traditional angiography and angioscopy procedures. The first phase of this project, which is partially described in this paper, focuses on the synthesis of aptamer-DOTA and aptamer-DTPA conjugates. Upon successful synthesis of the conjugates, they will be screened in the second phase of the project to find the best system for measuring thrombin in serum. Physiological conditions will be created to test the robustness of our system *in vivo*. Once the second phase has been completed, we will modify the aptamer to dually locate the blood clot and burst it.

Acknowledgements

I am grateful to Carleton University for providing the DNA synthesizer, the Gel electrophoresis setup, the UV-Vis spectrometer, and access to the 1.5 T MRI (Ottawa Hospital) through a collaboration with Dr. Eve Tsai. I am also deeply grateful to Erin McConnell and Dr. Maria DeRosa for their guidance and supervision.

Keywords

Blood clots; MRI; DNA; synthesis; thrombus; embolus; nanotechnology; caillots de sang; IRM; ADN, la synthèse; thrombus, embolie; nanotechnologies.

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Review of *Development Of An Aptamer-Based MRI Contrast Agent For Thrombin Detection*

The author must truly be commended on the strength of her work and effort on this elegantly crafted manuscript. It is inspiring to hear that this project was driven by secondary school student and her future is very bright in the medical sciences. Globally, the manuscript is articulate, robust and certainly well above the level of her training. Overall, this is an impressive manuscript and serves as a blueprint for her peers to aspire to.

The concept of harnessing nanotechnology for in essence 'personalized' or in this situation targeted medicine is promising. There are some minor laboratory-clinical "disconnects" that I have outlined but with these very small revisions this should proceed to publication.

Abstract

- Should use full form "magnetic resonance imaging (MRI)" before using short-form
- Is "blood clot" referring to arterial or venous thromboembolism – this should be clarified as it's unclear.
- The link from targeted MRI contrast and system of measuring serum thrombin is unclear. This jump requires clarification for the readership and is important.

Background

- Careful on clinical disconnect, MRI is not traditionally used clinically for detecting pulmonary embolism (PE) nor is invasive angiography conventional for diagnosing PE. CT-pulmonary embolism protocols standard and it is not invasive although does carry with it radiation. VQ scans are also used for the diagnosis of PE and does not require conventional contrast.
- In terms of "affecting healthy cells" it's unclear what the author is speculating on. Does she mean contrast induced nephropathy? This is usually CT contrast. MR traditionally uses gadolinium which is usually benign although can rarely lead to nephrogenic systemic fibrosis in dialysis patients. This is important to clarify if the author is suggesting targeted contrast may help avoid these possible, albeit rare side effects. This is alluded to in the background but should be clearer as it is the impetus for the current targeted contrast research.
- I caution the word "blood clotting" as it is colloquial and draw attention to the importance of clarifying venous or arterial venous thromboembolism – I presume VTE if you are referring to post-operative risks but would be well served to delineate.
- The sentence "Blood clotting during …" does not make clinical sense. VTE *prevention* isn't really technology based (i.e DVT prophlaxis with low dose heparin, mobilization, etc). And if you mean VTE detection, although angiography/angioscopy is 'classically' the gold standard, it is rarely, if ever used for detection. CT angiography and Doppler ultrasound are most commonly used and carry with it minimal side effects. This part needs to be clarified and would suggest the acknowledgement of the above followed by stating the emergence of MRI in both arterial/venous thromboembolism detection given minimal radiation as compared to CT angio. However, current gadollinum contrast carries with it risks such as NSF, etc.

Materials and Methods

- Is the DNA synthesizer protocol validated? If so, need to include the protocol citation.
- Is PAGE protocol validated? If so, need to include the protocol citation

Results

- For "known value" would recommend you cite this.

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